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Interleukin 23 regulates the functions of human decidual immune cells during early pregnancy

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ABSTRACT

Background: This study investigated the effects of interleukin 23 (IL-23) on the production of cytokines (IL-1, IL-4, IL-10, and IL-17), the differentiation of Treg/Th17 and STAT3 (i.e., signal transducer and activator of transcription 3) in human decidual immune cells (DICs) during early pregnancy.
Methods: DICs were treated with recombinant human IL-23 and an antibody against IL-23 subunit p19. The differentiation of Treg and Th17 cells was detected by flow cytometry. Levels of IL-23 receptor (IL-23R), STAT3, and phosphorylated STAT3 (pSTAT3) was examined by Western blot. The production of IL1, IL4, IL10, and IL-17 in DICs was measured by ELISA.
Results: Exogenous recombinant human IL-23 significantly promoted the differentiation of Th17 cells from DICs, while anti-IL-23 antibody significantly promoted the differentiation of Treg cells from DICs. Consistent with the differentiation of Th17 and Tregs cells, levels of IL-1β and IL-17 correlated positively with IL-23 treatment, and anti-IL-23 antibody increased the secretion of IL-4 and IL-10 from DICs. Levels of pSTAT3, but not STAT3 or IL-23R, were significantly elevated by recombinant IL-23 treatment; anti-IL-23 antibody significantly decreased the levels of pSTAT3 and IL-23R in DICs.
Conclusions: IL-23 mediates the differentiation of Th17 and Treg cells and the production of associated cytokines in DICs. The potential mechanism likely involves the STAT3 pathway.

1. Introduction

Interleukin 23 (IL-23) is a heterodimeric pro-inflammatory cytokine comprising IL-12 subunit p40 and IL-23 subunit p19. IL-23 is secreted by activated dendritic cells and macrophages [1], and is a crucial member in the innate immune system. Moreover, IL-23 facilitates the expansion and maintenance of T helper 17 (Th17) cells—it can promote the differentiation of Th17 cells, and within the Th17 cells, the production of IL-17 via IL-23R (IL-23 receptor) and STAT3 (signal transducer and activator of transcription 3) [2]. IL-17 is the dominant pro-inflammatory cytokine produced by Th17 cells. It has pleiotropic effects such as inducing the release of other cytokines that lead to proinflammatory processes and neutrophil-mobilization [3]. IL-23 and IL-17 have been implicated in the pathogenesis of many autoimmune diseases, such as inflammatory bowel diseases, psoriatic arthritis, and spondyloarthritis [4–6].

An embryo may be considered as a semi-allograft to the mother, and will be rejected by the mother's body if there is an imbalance in immunological tolerance [7], similar to the mechanisms of pathogenesis in autoimmune diseases. The decidua of the uterus is importantly involved in alterations in the immune response during key events such as blastocyst implantation, immunological tolerance, and trophoblast invasion, specifically through changes in cytokine expression and leukocyte cell recruitment [8,9].

Unexplained recurrent miscarriage (URM) refers to 2 or more pregnancy losses before the twentieth week of gestation [10]. The precise causes of URM are largely unknown. Recent studies suggested that IL-23 and IL-17 are involved in the pathogenesis of URM [11,12]. It is well established that there is a balance between Th1/Th2 and the regulatory T cell (Treg)/Th17 in the maternal–fetal interface, in which these cells secret cytokines including IL-1β, IL-4, IL-10 and IL-17. These cytokines form a complex network that is responsible for the maintenance of the pregnancy. An imbalance in these cytokines may result in spontaneous abortion [13,14].

Many studies have reported that URM patients appear to have a remarkably high amount of Th17 cells in the peripheral blood [12,15] and decidua [16]. Decidual immune cells (DICs) are a

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mixture of T cells, natural killer cells, macrophages and dendritic cells in the maternal–fetal interface [19]. However, the roles of IL-23 in DICs have rarely been studied.

In the present study, to improve understanding of URM causes we investigated effects of recombinant human IL-23 and IL-23 antibody on the production of cytokines (IL-1β, IL-4, IL-10 and IL-17) and the level of STAT3 in DICs from early pregnancy.

2. Materials and methods

2.1. Human decidual tissue collection

The Ethics Committee of First Affiliated Hospital of Guangxi Medical University (Nanning, China) approved this study. Each participant provided written informed consent.

The decidual samples were collected from 15 healthy pregnant women who sought an induced abortion at the Outpatient Department of the Gynecology Clinic at First Affiliated Hospital of Guangxi Medical University between January 2015 and May 2015. The age of the patients was 27.05 ± 2.80 years and gestational age was 51.80 ± 5.03 days. All pregnancies were determined intrauterine by ultrasound. None of the women were given treatment, including misoprostol or mifepristone, before the induced abortion, which was carried out by vacuum aspiration.

Decidual tissues from the aborted products were immediately collected in a mixture of 50% Dulbecco's modified Eagle's medium and 50% Ham's F-12 medium (DMEM/F12; Invitrogen, Life Technologies, Grand Island, NY) with antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin) and washed in sterile phosphate-buffered saline (PBS) before the isolation of DICs.

2.2. Isolation and culture of primary DICs

Isolation of DICs was performed as previously described [17]. Briefly, decidual tissues were cut into 1-mm^3 pieces and digested with 2 mg/mL collagenase IV (Sigma, St. Louis, MO) and DNase I (Sigma) at 37 °C for 1 h. The cell suspension was successively passed through 100, 300, and 400 mesh screens and centrifuged at 600 g in a discontinuous Percoll gradient. DICs that ranged in a density from 1.056 to 1.077 g/mL were recovered and cultured in 6-well plates in DMEM/F12 supplemented with 10% fetal bovine serum (Gibco, Life Technologies), 100 U/mL penicillin, and 100 mg/mL streptomycin (complete medium) with 5% CO_2 at 37 °C. In consideration of the phenotype alterations in different subjects, DICs from different patients were not mixed.

2.3. Treatment of human first-trimester DICs

DICs were seeded into 6-well plates (1.0×10^6/well) that were pretreated with 10 μg/mL purified anti-CD3 antibody (functional grade, eBioscience, San Diego, CA) overnight at 4 °C. To examine the effect of IL-23 on DIC cells, the wells with DICs were divided into 3 groups. The IL-23 group was treated with 3 ng/mL IL-23 protein (Peprotech, Rocky Hill, NJ). The anti-IL-23 group received 0.4 μg/mL anti-IL-23 antibody (eBioscience). The control group received no treatment. Each treatment was performed in triplicate. Moreover, to stimulate T lymphocyte differentiation *in vitro*, DICs of the 3 groups were cultured in the presence of 3 μg/mL anti-CD28 antibody (eBioscience) and 30 ng/mL IL-2 (Peprotech) in the culture medium.

2.4. Flow cytometry

After treatment for 24 h, 2×10^6 DICs were collected, washed with PBS and cultured in 1 mL of complete medium containing phorbol 12-myristate 13-acetate (PMA; 25 ng/mL) and ionomycin (1 μg/mL) for 5 h. For labeling the Th17 cells, DICs were stained using PerCP/Cy 5.5-conjugated anti-CD4 for 30 min at 4 °C in the dark, then washed with PBS and resuspended in Fix/Perm buffer (BD Pharmingen, San Jose, CA, both) and incubated for 30 min at 4 °C in the dark. After washing with Perm buffer twice, intracellular staining was performed using phycoerythrin (PE) anti-human IL-17 (BD Pharmingen) for 30 min. For Treg analysis, DICs were stained for PerCP-Cy5.5- conjugated anti-CD4, PE-conjugated anti-CD25, and Alexa Fluor 647-conjugated anti-FoxP3 (BD). All stained cells were acquired via a BD flow cytometer with CellQuest software and analyzed using Flowjo software (Treestar, Ashland, OR).

2.5. Enzyme-linked immunosorbent assay (ELISA)

After treatment for 12, 24 and 36 h, -the amount of culture medium of the DICs were collected and centrifuged at 450 × *g* for 5 min. The supernatant was collected to measure the levels of IL-1β, IL-2, IL-4, and IL-17 by ELISA in accordance with the manufacturer's instructions (YUANYE, Shanghai, China). All samples were measured in duplicate. The sensitivity of the ELISA kit was >1 pg/mL for IL-1β and IL-4, and >0.1 pg/mL for IL-10 and IL-17.

2.6. Western blot

After treatment for 24 h, DICs were washed in cold PBS and lysed in RIPA lysis buffer (Beyotime, Shanghai, China) for 30 min at 4 °C. The cell lysate was collected by centrifugation at 12 000 × *g* for 20 min at 4 °C. The protein concentration was determined by a nucleic acid protein detector (ThermoScientific, Waltham, MA). A total of 100 μg proteins were separated by 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (PVDF, 0.45 μm, Millipore, Billerica, MA). The membranes were blocked using 0.5% bovine serum albumin for 2 h, and incubated with the appropriate primary antibodies including STAT3 (1:2000, Cell Signaling Technology, Danvers, MA); pSTAT3 (1:2000; Cell Signaling Technology); IL-23R (1:500; Novus Biologicals, Littleton, CO); IL-17R (1:1000; Novus Biologicals); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:5000; Vazyme, Nanjing, China), overnight at 4 °C. After 2 washes, the membranes were incubated with the appropriate fluorescence-labelled secondary antibody (1:10,000, Licor, Lincoln, NE) for 1 h. The protein bands were detected using a Sweep membrane apparatus (LI-COR). The levels of proteins were analyzed with Image J software (National Institute of Health) and determined relative to GAPDH.

2.7. Statistical analysis

Data are presented as mean ± standard deviationStatistical comparisons were performed using one-way analysis of variance (ANOVA) or *t*-test with SPSS software (version 16.0; SPSS, Chicago, IL). Comparisons between 2 sets of data were performed by post hoc analysis. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Percentage of Th17 and Treg cells in DICs after treatment with IL-23 and anti-IL-23 antibody

After treatment with IL-23 and anti-IL-23 antibody, the percentages of Th17 ($CD4^+IL\text{-}17A^+$) and Treg ($CD4^+CD25^+FoxP3^+$) T cells in DICs were compared. The percentages of Th17/$CD4^+$ in the DICs of the control, IL-23, and anti-IL-23 groups were 1.31 ± 0.39%, 2.38 ± 0.73%, and 1.03 ± 0.27%, respectively (Fig. 1A). The percentages of Treg/$CD4^+$ in the DICs of three groups were

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